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Abstract: In previous research, we successfully cryopreserved intact human articular cartilage on its bone base with high chondrocyte viability using a vitrification protocol that entailed sequential exposure to several cryopreserving agents (CPAs) at lowering temperatures resulting in a high final concentration of CPA. The CPA must be removed from the cartilage at warming due to its toxicity to cells in the cryopreserved tissue and the post-transplant adjacent tissues. The current experiment explores the relationship between removal solution volume and time required for complete removal of CPA from bone-cartilage samples.
Osteochondral dowels of 10mm diameter from five patients undergoing total knee arthroplasty were vitrified using our protocol resulting in 6.5M CPA within the matrix. In the primary experiment, the warmed dowels were immersed in 10ml of X-VIVO for 30min and this was repeated 5 times (the last wash being 5 min only). Removal solution osmolality was recorded at various times and compared to controls of pure X-VIVO. Changes in removal solution osmolality over time were normalized to tissue volume. In a secondary experiment, the procedure was repeated using double the volume of removal solution (20ml X-VIVO).
Results showed a rapid change in the osmolality of the removal solution indicating a rapid efflux of CPA from cartilage. The efflux rate decreased with time and during subsequent immersions until equilibrium was reached during the 4th immersion indicating effectively complete removal of CPA. Doubling the amount of removal solution demonstrated the effective removal of CPAs by the third immersion.
The results of this study yield a practical relationship between the amount of removal solution and the time and number of immersions required to remove CPA from the transplantable tissue.

Clinical efflux of cryoprotective agents from vitrified human articular cartilage

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Introduction

Osteoarthritis is a massive burden on the health care system owing to its negative impact on quality of life and functional productivity of those affected. Progressive joint deterioration occurs with large cartilage defects[7]. Osteochondral allografting is one treatment option that can treat the largest defects[8; 12; 13; 26] with advantages over synthetic resurfacing techniques such as joint replacement because as biologic implants, allografts can integrate with the host tissue and function indefinitely. Unfortunately, fresh allografting is limited by tissue availability, difficultly matching the size and shape of the required cartilage tissue, the risk of infection transmission, and the necessity of transplantation within 48 to 72 hours.

To overcome these obstacles, investigations into effective methods of storing cartilage have been pursued. Hypothermic storage of allografts in 4 °C can provide storage for 28-42 days but cell deterioration begins after 7-14 days[5; 10; 29]. Classical cryopreservation methods using low concentrations of CPAs and controlled freezing showed some success when applied to chondrocytes in isolation[24], but resulted in low cell recovery when applied to complex articular cartilage tissue[19]. Recently, vitrification studies by various groups using high concentrations of CPAs and rapid cooling resulted in excellent cell recovery in pig articular cartilage removed from the bone[11], thin rabbit articular cartilage[25] and thin human articular cartilage removed from bone[27]. Most recently, we have developed a vitrification protocol that successfully cryopreserves intact human articular cartilage on a bone base with approximately 75% cell recovery as determined by membrane integrity dyes, a metabolic

assay and a functional assay that demonstrates the vitrified cells can produce cartilage specific collagen II and sulfated glycosaminoglycans[17]. Cryopreservation protocols are usually referred to as “vitrification” protocols if they involve high concentration of CPAs so that vitrification is the likely mechanism of cell protection, whether or not actual complete vitrification of the tissue has been confirmed. In the case of our protocol, we have verified that the final vitrification solution vitrifies, and does not devitrify, at the relevant cooling and warming rates, as confirmed by visual inspection for ice[28]. The cartilage cryopreserved with our vitrification protocol has the potential to be tissue banked and transplanted.

The use of CPAs in sufficient concentration to vitrify biologic tissues can result in cellular toxicity through different mechanisms. For example, dimethyl sulfoxide (Me_2SO) alters the cell membrane permeability by blocking the Na^+ and Ca^{2+} ion channels[9; 15], alters mitochondrial membrane potential[31], and induces cellular apoptosis[22]. Glycerol causes mitochondrial dysfunction by acting on the glycerol phosphate cycle[14], while ethylene glycol affects the ATP production, thereby affecting the biochemical functions of the cell[16]. To minimize toxicity, combinations of multiple CPAs have been used with each CPA at a lower concentration while still achieving an overall concentration sufficient to vitrify[6; 11; 21; 25].

Successful cryopreservation of intact human articular cartilage was recently achieved using a combination of 4 CPAs totaling 6.5M[17]. Prior to transplanting this tissue, the CPAs must be removed to avoid cellular toxicity of local cells and possible side effects

on the recipient patient. In our previous work, CPA permeation into cartilage off the bone was theoretically and experimentally studied[2; 3; 4; 20; 23], and for bone-cartilage samples, chondrocyte viability and function was experimentally assessed after cryopreservation, thaw and CPA removal[17]. Permeation and preservation of bone was not studied because the bone will get resorbed and incorporated into the host tissue post-transplantation and its viability is not required. However, for practical implementation of cryopreserved cartilage tissue in a clinical setting, it is important to understand trade-offs in different removal protocols for the entire bone-cartilage construct as will be used in practice. For this purpose, we studied the removal of CPAs from articular cartilage *in situ* in osteochondral dowels vitrified with our recently developed technique. To do this, we measured the change in removal solution osmolality over time. We hypothesized that a relationship between removal solution volume and time required for CPA removal could be identified to aid in the clinical application of this technique.

Material and Methods

Using a hand-held coring device, osteochondral dowels (OCD, full thickness articular cartilage on a bone base) were obtained from the weight bearing portion of distal femoral condyles of patients undergoing total knee arthroplasty in Edmonton, Canada. Patient information, including, age, gender, weight, height, medical diseases and smoking status was recorded. OCDs obtained were placed in a Dulbecco's Phosphate Buffer Saline (PBS) solution (pH7.1) and stored at 4 °C until they were either vitrified (an experimental group) or used as controls.

OCDs were vitrified using the 6.5M Me_2SO/glycerol/propylene glycol/ethylene glycol (DGPE) protocol developed by Jomha et al.[17] resulting in a concentration of 6.5M CPA within the matrix at the end of the protocol. Briefly, the dowels were sequentially placed in 6M Me_2SO for 1hr 30min at 0 °C; 6M glycerol and 2.4375M Me_2SO for 3hr 40min at 0 °C; 6M propylene glycerol, 2.4375M Me_2SO and 1.625 M glycerol for 3hr at -10 °C; and finally 6M ethylene glycol, 2.4375M Me_2SO, 1.625 M glycerol, and 0.8125M propylene glycerol for 1hr 20 min at -15 °C. Dowels were then placed in a storage solution containing 2.4375M Me_2SO, 1.625 M glycerol, 0.8125M propylene glycerol, and 1.625 M ethylene glycol in 15ml polypropylene BD Falcon Centrifuge Tubes (VWR International LLC). The tubes were then immersed in liquid nitrogen (LN_2) and kept at -196 °C until later use.

Five vitrified OCD samples, from five patients were selected randomly for the experiment. Tubes containing the vitrified dowels were removed from LN_2 and placed in a water bath of 37 °C until the solidified storage solution melted. The tubes were left in the water bath until used (time range: 5-7 minutes). The dowels were blot-dried using Kimwipe tissue (Kimberly-Clark, Roswell, GA), and the volume of each dowel was calculated using the average of heights measured from 3 different points.

The primary experiment consisted of an experimental and a control group. In the experimental group, three 10 mm diameter OCDs were immersed sequentially into five 15 mL conical centrifuge tubes, each containing 10ml X-VIVO at 4 °C for 30 minutes. The osmolality of the removal solution was recorded at 0, 1, 3, 5, 10 and 30 min. A

‘µOSMETTE’ micro-osmometer (Precision Systems, Natick, MA) was used to measure the osmolality of a 50µl sample of the removal solution. The extracted solution was replaced after each reading. A magnetic stirring bar (size equal to, or smaller than, 8mm x 1.5mm) was placed at the bottom of each tube to aid the mixing of the solution. The tubes were kept in a plastic rack, centered on a stirrer. After 30 minutes in one tube, the dowel was removed and dried, weighed and immersed in the next conical centrifuge tube containing 10 mL X-VIVO. Again, using the same procedure, the change in osmolality was measured at the same time intervals. This procedure was repeated 5 times in total with the exception of the last immersion, which lasted for only 5 minutes. After the last immersion, the dowels were dried and weighed.

The experimental control consisted of two fresh 10 mm diameter osteochondral samples from two different donors immersed in 10 mL X-VIVO with the experimental protocol performed in the same fashion with osmolality measurements done at the same time intervals. However, the control dowels were immersed sequentially into two X-VIVO tubes instead of five tubes.

In order to study the effect of increasing the volume of the removal solution on the rate of CPA efflux, a secondary experiment was completed in a similar fashion to the primary experiment using two 10mm diameter OCDs placed in subsequent removal solution washes of 20 ml of X-VIVO (as opposed to 10ml in the primary experiment).

The change in osmolality (measured osmolality minus original removal solution osmolality) was normalized to the calculated volume of the dowel (ΔOsmolality/ml dowel). The average and standard error of the measurements was recorded and plotted against time.

Results

Five vitrified osteochondral dowels and two fresh dowels from seven total knee arthroplasty patients were used for the experiment as described in the Materials & Methods section. The ages of the patients ranged from 55 to 77 (mean = 67.6). Of the 7 patients, 4 were males and 3 were females. The volumes of the dowels used for the primary and secondary experiments and for experimental controls were 0.57 ± 0.06mL, 0.56 ± 0.01mL, and 0.82 ± 0.01mL, respectively.

The results for the primary experiment (Figure 1), the secondary experiment (Figure 2) and the control samples (Figure 3) are shown below. In both experiments, the initial rate of change in osmolality of the removal solution per dowel volume was large in each wash. Subsequently, the rate of change in osmolality decreased with time and subsequent washes until a plateau was reached. The plateau was considered to be reached when the change in osmolality of the removal solution was less than the change in osmolality of the control samples. Immersing the fresh dowels in the control solution resulted in minimal changes in the osmolality of the removal solution (0 to 15.44 mOsmol/ mL).

The number of washes required to remove CPA from vitrified dowels differed between the experiments. In the primary experiment, the osmolality stabilized during the 4th

immersion. The 5th immersion confirmed that the plateau had been obtained (Figure 1). When the volume of the removal solution was doubled, osmolality stabilized during the third immersion. The 5th immersion was not carried out since no change in osmolality was evident during the 4th immersion (Figure 2). The ratio of the volume of dowel to the volume of removal solution was 1:17.4 in the primary experiment and 1:36.0 in the secondary experiment.

Discussion

An effective protocol to vitrify intact human articular cartilage has recently been developed by our group[17]. Removal of the CPAs from vitrified articular cartilage is mandatory before the clinical use of this tissue to limit the potential toxic effect of CPAs on the local cartilage cells and to the patient systemically. Interestingly, there are limited guidelines regarding safety levels of CPAs in the body. The American Association of Blood Banks (AABB) states: "*care should be taken not to exceed 1 mL of DMSO per kilogram of recipient weight per day administration*" when transplanting bone marrow cells[1]. Using this recommendation, the acceptable amount of CPA in a 70 kg person is 70 ml. With respect to transplantation of orthopaedic joint tissues, it is highly unlikely that it would even be possible to have 70ml of CPAs within the transplanted tissue given the relatively small volume of the transplanted tissue. Even though systemic toxicity is highly unlikely, it is important to remove as much CPAs as possible to limit local toxicity. Therefore we need to know how long it takes to remove essentially all of the CPA from the transplantable tissue.

The results of the experiment show the number of washes required to remove CPAs from vitrified dowels using multiple immersions in X-VIVO, each for 30 minutes based on the volume of the diluting solution. In the primary experiment, four washes were required to remove all CPA from vitrified cartilage. In the secondary experiment, when the ratio of volume of dowel to volume of removal solution was decreased, only three washes were required. The efflux was rapid in the first wash for both experiments. The efflux rate then decreased with subsequent washes until a plateau was reached indicating the efflux of most of the CPA. Based on the information provided here, it is reasonable to assume that essentially all of the CPAs are removed after 4 washes in X-VIVO for 30 minutes when the ratio of tissue to removal solution is $1:36 < x \leq 1:17$. Only 3 washes are required when the ratio is less than or equal to 1:36.

As a further check that the majority of CPA has been removed from the dowel by the end of all immersions, a rough mole balance can be performed. It is important to note for this mole balance that the dowel is made up of both cartilage and bone and that these two biomaterials have different capacities to hold CPA. The number of moles of CPA removed by the end of all immersions can be found by summing the end average osmolalities (this assumes that the removal solutions are dilute, which is a good assumption), dividing by 1000 to convert units from osmoles per litre to osmoles per mL) and multiplying by the number of mLs in the removal solution (since we need moles per total removal solution). For example, in the case of the primary experiment, the total CPA found in the removal solutions is 163.4 + 41.5 + 26.7 + 17.8 + 15.5 = 265 mmolal or 0.265 mmoles/mL or 2.65 mmoles in 10 mL. This 2.65 mmoles of CPA were

originally in the 1 mL of dowel. Note that all osmolality data is per mL of dowel in order to be able to average different dowels with different volumes. To convert this mmoles per total dowel volume into a CPA loading molarity, requires knowledge of what fraction of the dowel holds solution. Assuming that the native water content can be used as a rough measure of fraction of the biomaterial that can be taken up by CPA solution, we use a water content (or solution fraction) for human femoral condyle articular cartilage of 77.6 %[23] and for human femur bone of 12.8%[30]. Then the original sample molarity is computed by the equation below:

$$Sample\ molar\ CPA = \frac{2.65\ mmoles}{sample\ solution\ volume\ in\ mL}$$

$$= \frac{2.65\ mmoles}{1\ mL\ [cartilage\ fraction\ \times\ 0.776 + bone\ fraction\ \times\ 0.128]}$$

The dowels used in this experiment were not separated into their constituent cartilage and bone portions. However, if we assume that the dowel started with 6.5 Molar CPA in the solution space, we can find what cartilage fraction satisfies the above equation. From the equation it was found for the primary experiment that if the samples were on average 43% cartilage and 57% bone, the mole balance would be satisfied and the amount of CPA removed during the immersions would equal all the CPA that started in the dowel. Since the average dowel height in the primary experiment was 7.3 mm, this would mean that the average cartilage thickness would be predicted to be 3.1 mm if the mole balance was satisfied. A similar rough mole balance for the secondary experiment indicates that the

259 average cartilage thickness would be predicted to be 1.8 mm if the mole balance was
260 satisfied. The actual thicknesses of the cartilage portion of the OCDs in this study were
261 not measured; however cartilage thickness measurements at various locations within
262 osteochondral dowels from the same tissue source (but different patients) in a separate
263 study yielded thicknesses ranging from 1.7 mm to 3.3 mm. Since the thicknesses
264 predicted by the mole balances give a reasonable estimation of the actual human cartilage
265 thickness in experiments, we can conclude that a rough mole balance supports the
266 assertion that the majority of CPA is removed after completion of all immersions.
267
268 It is interesting to note from a practical standpoint that although it takes 9.5 hours to load
269 the CPA into the dowels to vitrify them, with the removal procedure we propose it takes,
270 at most, only 2.0 hours to remove the CPA. This is because the removal happens at a
271 higher temperature and CPA permeation rates are exponentially dependent on
272 temperature and also because replacing the removal solution with fresh CPA-free
273 removal solution every 30 minutes maintains a high driving force for removal.
274
275 In conclusion, the amount of CPA within the cartilage matrix after vitrification is
276 acceptable for transplantation with current guidelines. Removal of essentially all of the
277 CPA is easily obtained with 3 or 4 washes in X-VIVO for 30 minutes each depending on
278 the tissue to removal solution volume ratio.
279
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284
285

Figure Legends

Figure 1: Graphs showing the change in osmolality of the removal solution in the primary experiment (immersion in subsequent washes of 10 ml of X-VIVO). It is important to note the rapid osmolality change early on that decreases with time with a resultant plateau during the 4^{th} immersion. N = 3 $\pm$ SEM.

Figure 2: Graphs showing the change in osmolality of the removal solution in the secondary experiment (immersion in subsequent washes of 20 mL of X-VIVO). Once again there was a rapid increase in the osmolality change at early time points. The osmolality change plateaued during the 3^{rd} immersion. N = 2 ± SEM. (Note that for N = 2, the SEM simply shows exactly the range of the two results.)

Figure 3: Graphs showing the change in osmolality of the control samples. Note that there was minimal change in osmolality at any time point. N = 2 ± SEM. (Note that for N = 2, the SEM simply shows exactly the range of the two results.)

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Conflict of Interest

The authors have no conflict of interest.

Figure 1

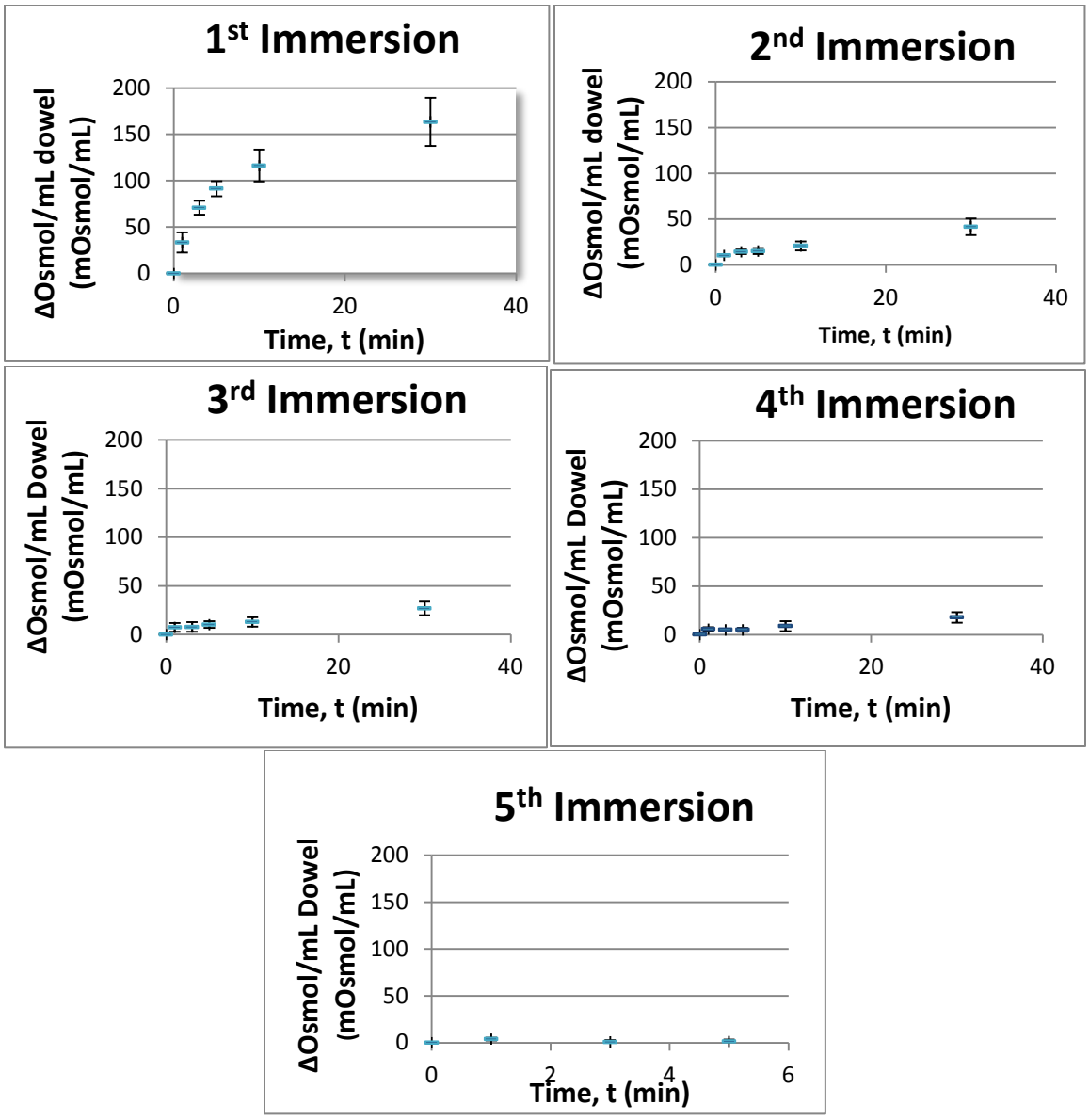

1st Immersion
ΔOsmol/mL dowel (mOsmol/mL)
200
150
100
50
0
0
20
40
Time, t (min)
2nd Immersion
ΔOsmol/mL dowel (mOsmol/mL)
200
150
100
50
0
0
20
40
Time, t (min)
3rd Immersion
ΔOsmol/mL Dowel (mOsmol/mL)
200
150
100
50
0
0
20
40
Time, t (min)
4th Immersion
ΔOsmol/mL Dowel (mOsmol/mL)
200
150
100
50
0
0
20
40
Time, t (min)
5th Immersion
ΔOsmol/mL Dowel (mOsmol/mL)
200
150
100
50
0
0
2
4
6
Time, t (min)

Figure 2

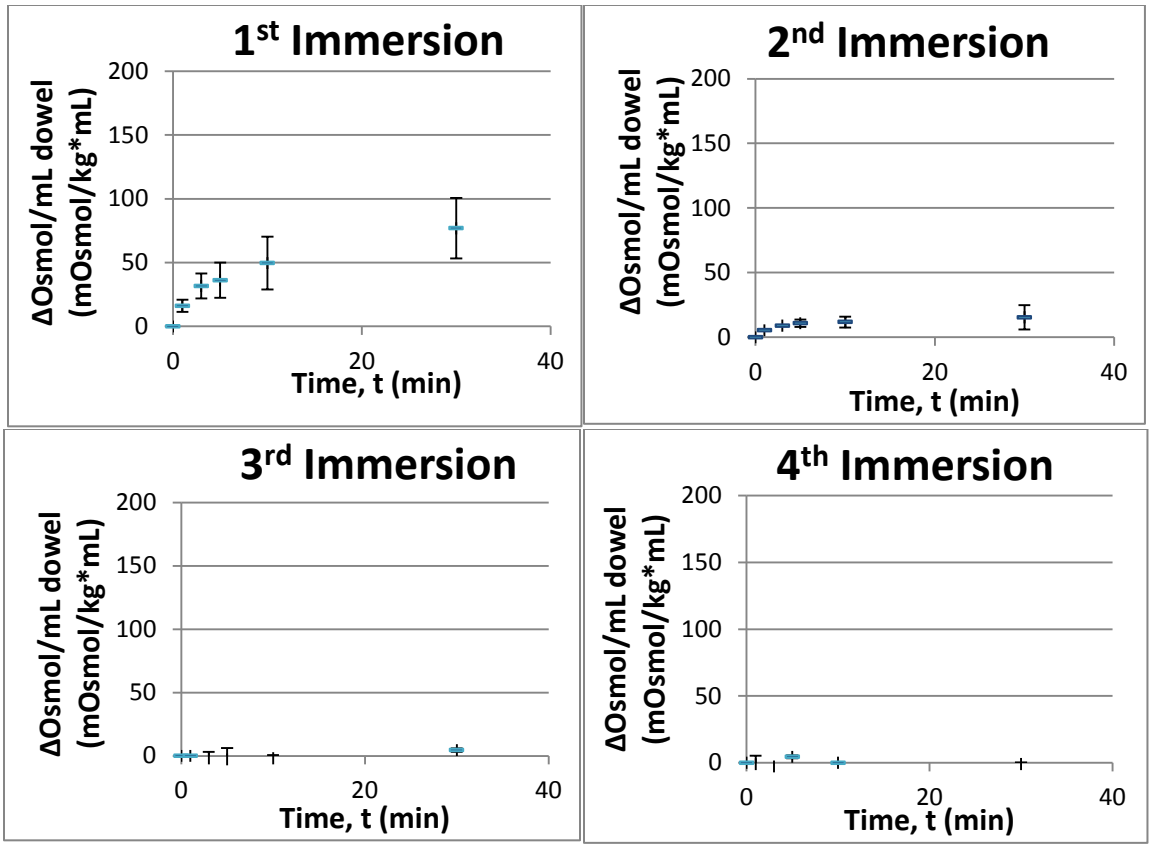
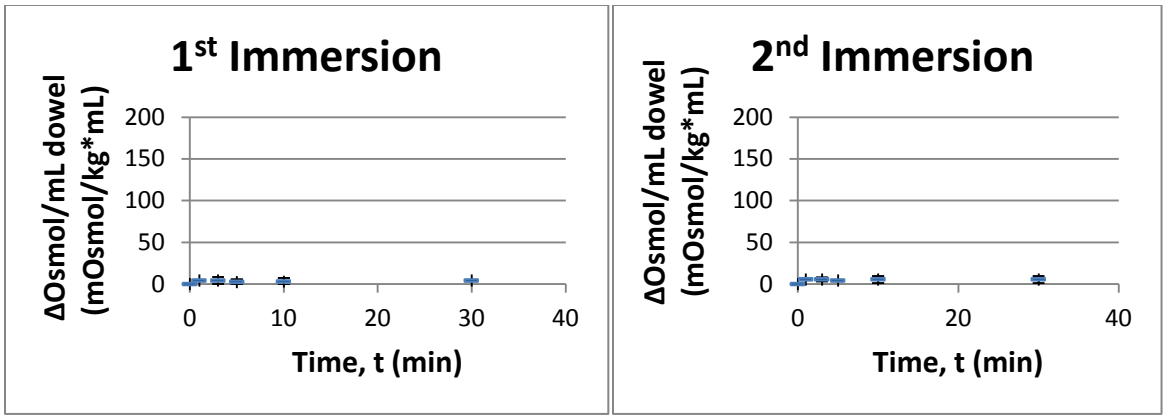

1st Immersion
2nd Immersion
3rd Immersion
4th Immersion
ΔOsmol/mL dowel (mOsmol/kg*mL)
Time, t (min)
0
50
100
150
200
0
20
40

Figure 3


1st Immersion
ΔOsmol/mL dowel (mOsmol/kg*mL)
200
150
100
50
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10
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30
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Time, t (min)
2nd Immersion
ΔOsmol/mL dowel (mOsmol/kg*mL)
200
150
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0
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Time, t (min)